

Selective suppression of Toll-like receptor 4 activation by chemokine receptor 4

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Abstract The response of Toll-like receptor 4 (TLR4) to lipopolysaccharide (LPS) is thought vital for resisting infection. Since aberrant TLR4 signaling may initiate inflammatory conditions such as the sepsis syndrome, we sought a component of normal cells that might provide local control of TLR4 activation. We found that antibodies that block chemokine receptor 4 (CXCR4) function enhanced TLR4 signaling, while increased expression of CXCR4 or addition of the CXCR4 ligand SDF-1 suppressed TLR4 signaling induced by LPS. These findings suggest that CXCR4 could exert local control of TLR4 and suggest the possibility of new therapeutic approaches to suppression of TLR4 function.

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1. Introduction

As expressed on leukocytes, endothelial cells and various parenchymal cells, Toll-like receptor 4 (TLR4) detects the products of microorganisms, such as lipopolysaccharide (LPS) [1–3], and endogenous substances, such as heparan sulfate and hyaluronic acid [4,5]. Following detection of these substances, TLR4 transduces signals leading to activation of NFκB, among other signaling pathways [6], that induce the expression of genes that incite inflammation and adaptive immunity. These responses sequester and clear microorganisms and heighten resistance of the host upon re-infection [7,8].

The inflammatory and immune responses triggered by TLR4 must be vital to host defense as they are induced by nanomolar concentrations of LPS and mice with mutations that preclude function or expression of TLR4 have marked susceptibility to infection with Gram negative bacteria [2]. However, the func-

tion of TLR4 is not always beneficial. Widespread activation of TLR4 gives rise to the “sepsis syndrome” characterized by shock, multi-organ failure and death [9]. Aberrant activation of TLR4 has also been implicated in chronic inflammatory diseases such as atherosclerosis [10], asthma [11], osteoporosis and even obesity [12].

If activation of TLR4 is to benefit the host and the systemic changes in physiology characteristic of sepsis or chronic inflammation are to be avoided, activation should be limited to sites of infection (where microorganisms and their antigens are present) and constrained at remote locations, where microorganisms and antigens are lacking. To a certain extent, such local control of TLR4 might be achieved through the function of the secretory leucoprotease inhibitor (SLPI), a protein that blocks TLR4-activated intracellular signals [13,14]. However, SLPI is found mainly in secretions on epithelial cell surfaces and thus would not prevent unwanted activation of TLR4 in most tissue spaces. Given the need to control activation of TLR4 at sites remote from infection, we sought normal cellular constituents that might regulate TLR4 function.

TLR4 exists and functions as a receptor complex, which in addition to TLR4 includes MD2 and CD14 [15]. Other cell surface proteins, including heat shock proteins (HSP) 70 and HSP90, growth and differentiation factor 5 and chemokine receptor 4 (CXCR4), can participate in TLR4 signaling [16,17]. How these proteins function as a putative “LPS activation cluster” is not known, although HSP70 and HSP90 may do so by processing LPS [18,19] or by acting directly on TLR4 [20–22]. Of these potential regulators, CXCR4 has a dual function as, independent of any impact on TLR4, it promotes migration of antigen presenting cells outside of blood vessels and into secondary lymphatic organs. We reasoned that this dual function of CXCR4 and the positioning between innate and adaptive immunity [23,24] would make CXCR4 a good candidate for an intrinsic regulator of TLR4 function. We report the testing of that concept.

2. Materials and methods

2.1. Sources of reagents and antibodies

LPS from *Escherichia coli* was obtained from Sigma–Aldrich (St. Louis, MO). A neutralizing monoclonal antibody specific for chemokine receptor 4 was from R&D Systems Inc. (Minneapolis, MN).

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Abbreviations: TLR, Toll-like receptor; CXCR4, chemokine receptor 4; LPS, lipopolysaccharide; SDF-1, stromal-cell derived factor-1; NFκB, nuclear factor kappa B; SLPI, secretory leucoprotease inhibitor; HSP, heat shock protein

Monoclonal anti-CXCR4 and control mouse IgG2a κ conjugated to allophycocyanin (APC) were from BD Biosciences (San Diego, CA). Fluorescein isothiocyanate (FITC)-conjugated antibodies were from Southern Biotechnology Association (Birmingham, Alabama). Anti-mouse TLR4/MD-2 was from BioSource (Camarillo, CA). Pertussis Toxin (*Bordetella pertussis*) and Pertussis Toxin-B (apoenzyme) were from CalBiochem (La Jolla, CA). All materials used in cell culture were certified endotoxin free or were treated with endotoxin removal resin and tested by the Limulus amoebocyte lysate assay gel clot method (Seikagaku, Falmouth, MA) to assure absence of detectable endotoxin.

2.2. Plasmid construction

Expression vectors encoding components of the TLR4 complex were prepared as follows. Total RNA isolated from the murine macrophage cell line RAW 264.7 (American Type Culture Collection, Manassas, VA) was used to generate cDNA using the "1st Strand cDNA Synthesis Kit" (Roche, Indianapolis, IN) for RT-PCR (AMV) with oligo(dt) primers, according to the manufacturer's recommended protocol. The resulting pool of cDNA was used as a template in polymerase chain reactions (PCRs) to amplify sequences encoding TLR4, MD2 and CD14, using "Expand High Fidelity" polymerase (Roche). TLR4 was amplified using the primers: TLR4 Forward 5'-CGC GGA TCC AGG ATG ATG CCT CCC TGG CTC-3' and TLR4 Reverse 5'-

GGC GGT ACC TCA GGT CCA AGT TGC CGT TTC-3'. MD2 was amplified using MD2 forward 5'-CCG GAA TTC ATC ATG TTG CC-3' and MD2 reverse 5'-CCG GAA TTC CTA ATT GAC ATC ACG-3'. CD14 was amplified using CD14 forward 5'-CCG GAA TTC ACC ATG GAG CGT GTG CTT GGC-3' and CD14 reverse 5'-CCG GAA TTC TTA AAC AAA GAG GCG ATC TCC TAG-3'. PCR products were cloned into eukaryotic expression plasmids (Invitrogen, Carlsbad, CA): TLR4 into pcDNA3.1, MD2 into pcDNA3.1/Hygro CD14 into pcDNA4/myc-His with zeocin resistance. Cloned sequences were screened for correct orientation and sequence. A NF κ B-firefly luciferase reporter plasmid was obtained from Dr. Carlos Paya (Mayo College of Medicine, Rochester, MN). Control *Renilla*-luciferase reporter plasmid was pTK-*Renilla* (Promega, Madison, WI). The YFP-CXCR4 expression plasmid was prepared by cloning the CXCR4 coding sequence [25] into the pEYFP-C1 vector (Clontech, Palo Alto, CA) with the vector encoded yellow fluorescent protein appended in frame with the carboxy-terminus of the CXCR4 coding sequence.

2.3. Cell cultures

HEK 293 cells (human embryonic kidney cells) from American Type Culture Collection (ATCC) were cultured in DMEM containing 10% fetal bovine serum, penicillin and streptomycin. HEK 293

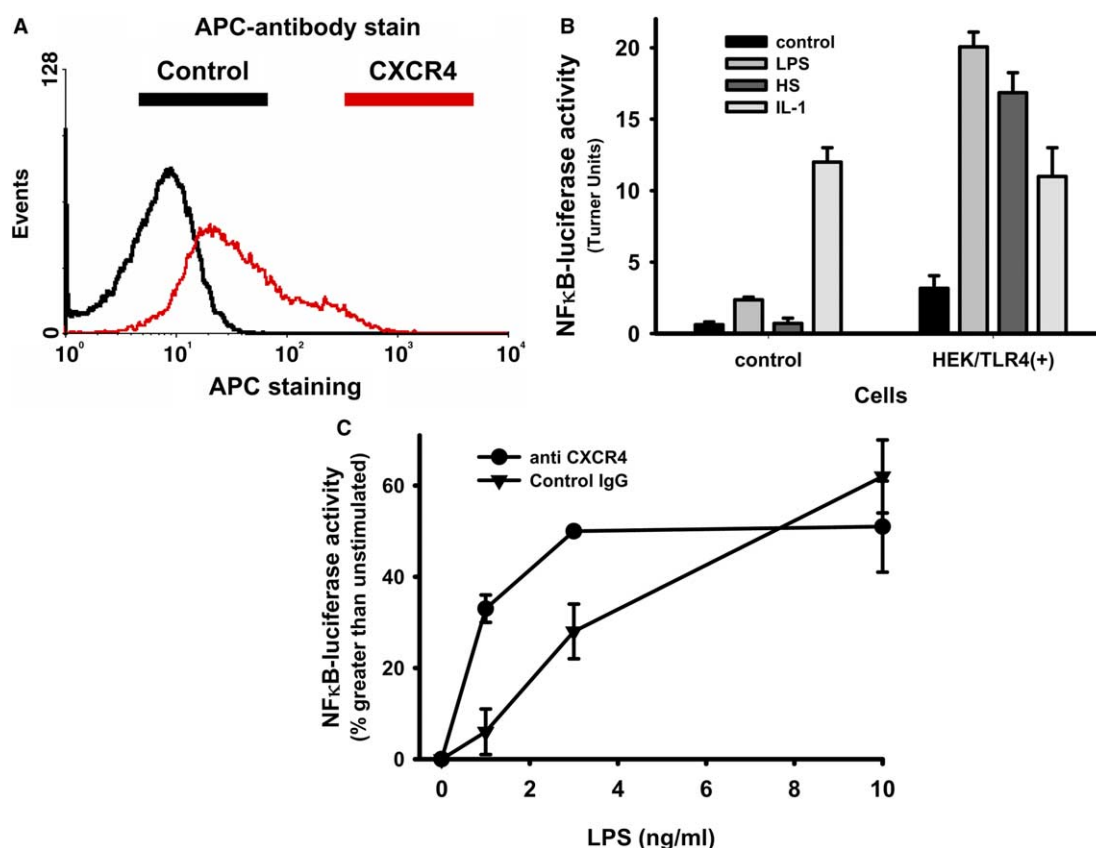


Fig. 1. Activation of TLR4 in HEK 293 cells. Whether CXCR4 modifies TLR4 activation was tested by measuring the impact of perturbing CXCR4 using monoclonal antibodies and testing the effect of this treatment on TLR4 activation by LPS in HEK 293 cells. For this end, HEK 293 cells were stably transfected with murine TLR4, MD2 and CD14 expression plasmids and cell lines that express these proteins were selected (HEK/TLR4(+) cells). The cells were then characterized and tested as shown in the figure. (A) CXCR4 expression on HEK/TLR4(+). CXCR4 expression on HEK/TLR4(+) cells was determined by flow cytometry using anti-CXCR4 or control APC-conjugated monoclonal antibodies. The results show that HEK/TLR4(+) cells express CXCR4. (B) Stimulation of HEK 293 cells by LPS and heparan sulfate. HEK/TLR4(+) cells or control HEK 293 cells (that lack TLR4) were transfected with NF κ B-firefly luciferase and internal control *Renilla*-luciferase reporter plasmids and then tested for response to 10 ng/ml of LPS, 10 μ g/ml of heparan sulfate, or 10 ng/ml recombinant human IL-1 α . NF κ B-activated luciferase expression was measured 4 h after stimulation, results shown are means of triplicate wells. These results, which are representatives of three experiments, show that HEK/TLR4(+) cells respond to LPS and heparan sulfate. (C) Impact of CXCR4 on activation of TLR4 by LPS. To determine if CXCR4 affects the ability of LPS to activate TLR4, HEK/TLR4(+) cells were transfected with NF κ B- and control-luciferase reporter plasmids and then treated with 25 μ g/ml anti-CXCR4 monoclonal antibodies or control antibodies of the same isotype for thirty minutes, after which the indicated amount of LPS was added. HEK/TLR4(+) response was measured by NF κ B-activated luciferase expression determined six hours after addition of LPS. The anti-CXCR4 antibodies enhanced responsiveness of HEK/TLR4(+) cells to LPS.

cells were transfected with TLR4, MD2 and CD14 expression plasmids using the Superfect transfection reagent (Qiagen, Valencia, CA), as suggested by the manufacturer for stable transfection. TLR4/MD2/CD14 expressing HEK 293 cells were selected using appropriate antibiotic selection medium and were then cloned by limiting dilution in the same medium. Control cells were prepared using empty expression vectors and identical transfection and selection procedures.

2.4. Stimulation of HEK 293 cells

HEK 293 cell lines stably expressing TLR4/MD2/CD14 were seeded into 24-well tissue culture plates (2×10^5 cells/well) and allowed to adhere at 37 °C overnight. The adherent cells were transfected with 0.1 µg pTK-*Renilla* luciferase and 0.1 µg NFκB-firefly luciferase using Superfect Transfection Reagent (Qiagen) and then cultured for 24 h at 37 °C

in 1 mL DMEM containing 0.5% fetal bovine serum. The cells were stimulated as indicated, washed with phosphate buffered saline and lysed with 150 µL Passive Lysis Buffer (Promega). The amount of *Renilla*- and Firefly luciferase in the cell lysates was assayed simultaneously using Dual-Luciferase Reporter Assay System (Promega) and a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Activation of NFκB-firefly luciferase reporter activity is given as the ratio of firefly luciferase to the internal control *Renilla*-luciferase activity, determined and expressed as the mean of triplicate wells.

2.5. Flow cytometry

Flow cytometric analysis was performed as described by Kodaira et al. [26]. HEK 293 cells were incubated with APC-conjugated anti-CXCR4 monoclonal antibodies and analyzed by FACScan using Cell-Quest software (Becton–Dickinson, San Jose, CA).

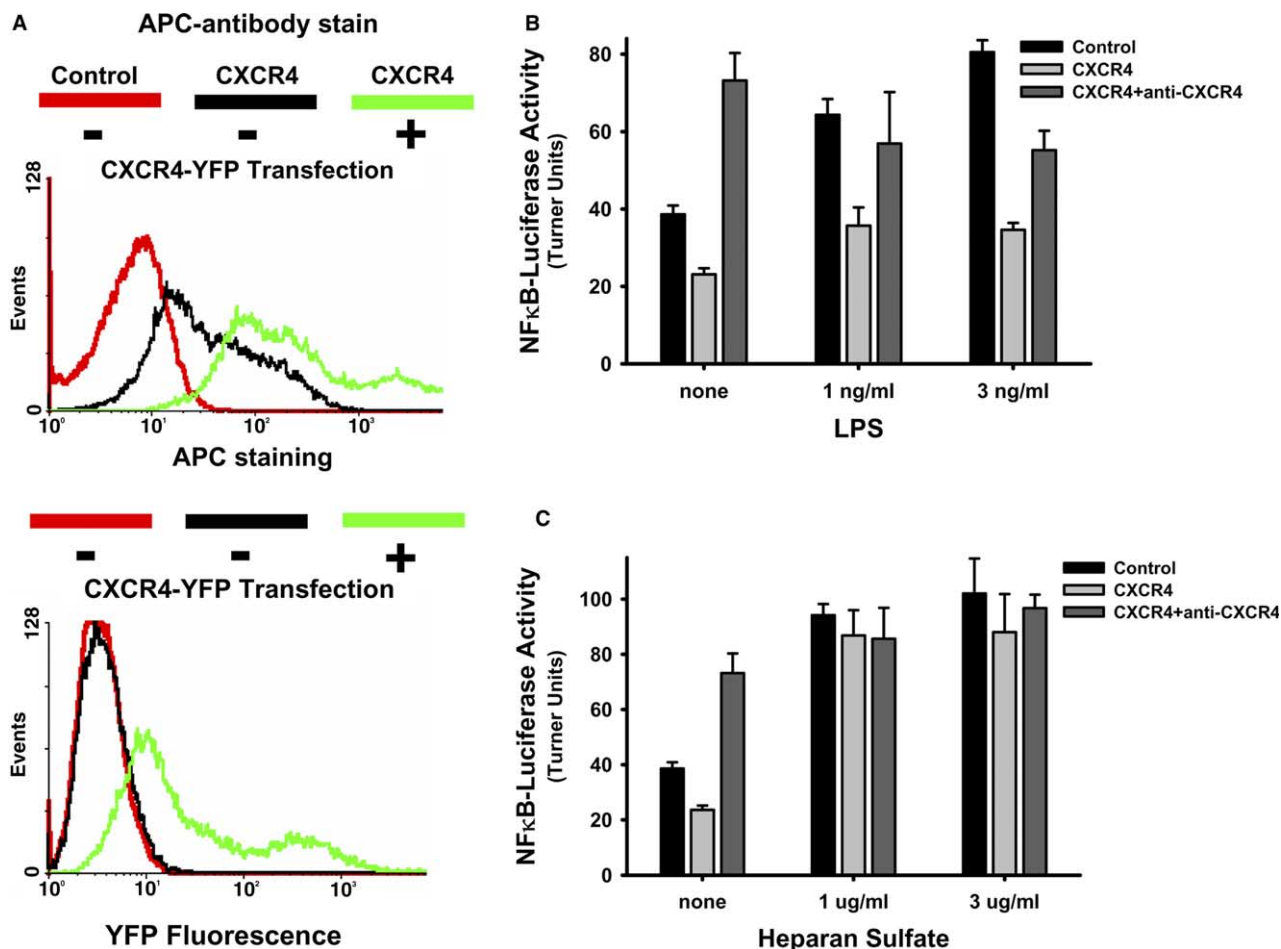


Fig. 2. Effect of enhanced expression of CXCR4 on TLR4 activation. To determine whether CXCR4 diminishes responsiveness to LPS, HEK/TLR4(+) cells were transfected with a vector encoding human CXCR4 fused to yellow fluorescent protein (CXCR4-YFP) or control expression plasmids, along with NFκB- and control-luciferase reporter plasmids and responsiveness to LPS was determined. (A) CXCR4 expression in HEK/TLR4(+) cells transfected with CXCR4-YFP. HEK/TLR4(+) cells were transfected with CXCR4-YFP vector and CXCR4 expression on the surface of the cells was determined using an anti-CXCR4 monoclonal antibody conjugated to APC (upper panel) or by directly measuring CXCR4-YFP fluorescence (lower panel). These results demonstrate that HEK/TLR4(+) cells transfected with CXCR4-YFP express CXCR4 at about 5-fold higher level than controls. (B) Enhanced expression of CXCR4 decreases responsiveness of HEK/TLR4(+) cells to LPS. HEK/TLR4(+) cells were transfected with CXCR4-YFP or control expression vectors along with NFκB- and control-luciferase reporter plasmids and responsiveness of the cells to LPS was determined. Some samples were treated with 25 µg/ml of anti-CXCR4 monoclonal antibodies (anti-CXCR4) prior to stimulation with LPS. HEK/TLR4(+) cells transfected with CXCR4 did not respond to LPS. HEK/TLR4(+) cells transfected with CXCR4 and treated with anti-CXCR4 antibodies activated the NFκB-luciferase reporter fully. These results indicate that cell surface expression of CXCR4 interferes with TLR4 activation by LPS. (C) Control of TLR4 signaling by CXCR4 is specific for LPS. To determine if suppression of TLR4 by CXCR4 is specific to LPS, HEK/TLR4(+) cells were transfected with CXCR4 or control expression plasmids and NFκB- and control-luciferase reporter plasmids and responsiveness to heparan sulfate, an endogenous activator of TLR4, was tested. CXCR4 expression, or neutralization of CXCR4 function with anti-CXCR4 monoclonal antibodies, did not affect activation of HEK/TLR4(+) cells by heparan sulfate. This result indicates that CXCR4 specifically interferes with activation of TLR4 by LPS.

3. Results and discussion

3.1. CXCR4 and the TLR4 response to LPS

Since CXCR4 is found in the “LPS activation cluster” [16,17] and can interact with other cell surface receptors, we asked whether it might influence activation of TLR4. To determine whether CXCR4 influences TLR4 activation by LPS, we developed a model using HEK 293 cells, which naturally express CXCR4 (Fig. 1A) but not TLR4 (not shown). The HEK 293 cells were made to express defined components of the TLR4 complex [15] by transfection with vectors encoding murine TLR4, MD2, and CD14 and selection of clones that stably express these proteins (HEK/TLR4(+)) cells, data not shown). HEK 293 cells transfected this way respond to LPS and heparan sulfate (Fig. 1B).

To determine whether CXCR4 influences activation of TLR4 by LPS, HEK/TLR4(+) cells were transfected with NF κ B-firefly luciferase and internal control *Renilla*-luciferase reporter plasmids and then the impact of inhibition of CXCR4 on TLR4 signaling was measured. As Fig. 1C shows, when HEK/TLR4(+) cells were treated with anti-CXCR4 antibodies that perturb the conformation and function of the receptor, responses to low concentrations of LPS (less than 3 ng/ml) were increased nearly 2-fold compared to HEK/TLR4(+) cells treated with control IgG. Anti-CXCR4 antibodies did not modify activation of HEK/TLR4(+) by higher concentrations of LPS (10 ng/ml). This result suggests that CXCR4 raises the threshold for TLR4 activation.

If CXCR4 inhibits activation of TLR4 by LPS, we reasoned that overexpression of CXCR4 might potentiate the inhibition. To test this possibility, we transfected HEK/TLR4(+) cells with a plasmid vector encoding human CXCR4 and the NF κ B- and control-luciferase reporter plasmids and tested responses to LPS. The HEK/TLR4(+) cells transfected with CXCR4 expressed that protein at 5-fold greater levels than controls (Fig. 2A) did not respond to LPS (Fig. 2B). To confirm that heightened expression of CXCR4 and not some other factor suppressed TLR4 signaling, we treated HEK/TLR4(+) cells that had been transfected with CXCR4 with anti-CXCR4 monoclonal antibodies and then stimulated the cells with LPS. As Fig. 2B shows, HEK/TLR4(+) cells transfected with CXCR4 and treated with anti-CXCR4 antibodies responded by activating the NF κ B-luciferase reporter, confirming that CXCR4 had suppressed TLR4 signaling.

To determine if CXCR4 must be activated to suppress stimulation of TLR4 by LPS, we tested whether increasing availability of SDF-1, the agonist for CXCR4 [27], would potentiate inhibition of HEK/TLR4(+) cell responses to LPS. As Fig. 3 shows, when HEK/TLR4(+) cells were stimulated simultaneously with LPS and SDF-1, the cells were indifferent to the presence of LPS at any concentration tested (Fig. 3A). SDF-1 by itself initiated a slight activation of the luciferase reporter, consistent with findings that CXCR4 is a weak stimulator of NF κ B [28], but this level of activation is far below that observed for LPS treatment alone. Treatment of HEK/TLR4 cells with SDF-1 did not alter expression of TLR4 (not shown), suggesting that signals delivered through CXCR4 strongly inhibit activation of TLR4 signaling by LPS.

We next asked whether the inhibition of TLR4 by CXCR4 is specific for LPS. To address this question, HEK/TLR4(+) cells transfected with CXCR4 and NF κ B- and control-lucif-

erase reporter plasmids were tested for response to heparan sulfate, an endogenous activator of TLR4 [4]. As shown in Fig. 2C, HEK/TLR4(+) cells responded fully to heparan sulfate despite overexpression of CXCR4, and treatment of HEK/TLR4(+) cells with anti-CXCR4 antibodies did not influence responsiveness to heparan sulfate. These results indicate that CXCR4 specifically constrains signaling by TLR4 induced by LPS. Failure of CXCR4 to inhibit activation of TLR4 by heparan sulfate has several potential explanations. Heparan sulfate may act somewhat differently on

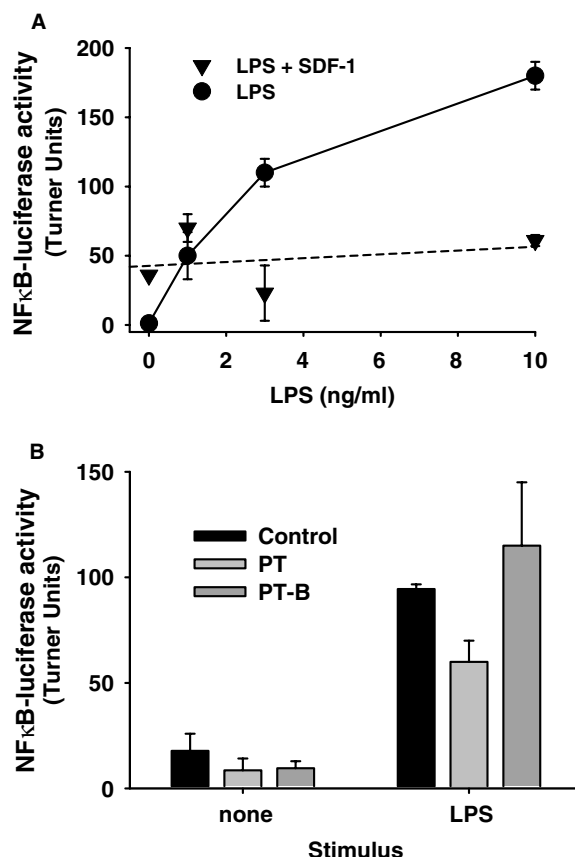


Fig. 3. Effect of signaling by CXCR4 on TLR4 activation. To determine if the inhibitory effect of CXCR4 on TLR4 activation requires signaling by CXCR4, HEK/TLR4(+) cells were transfected with NF κ B- and control-luciferase reporter plasmids and treated with the CXCR4 agonist SDF-1, or with pertussis toxin (PT), an inhibitor of CXCR4 signaling, prior to stimulating the cells with LPS. (A) CXCR4 signaling inhibits TLR4 activation by LPS. To determine if CXCR4 signaling inhibits TLR4 activation by LPS, HEK/TLR4(+) cells were transfected with NF κ B- and control-luciferase plasmids and treated with 50 nM SDF-1, an agonist of CXCR4, and stimulated with the indicated concentrations of LPS. Stimulation of CXCR4 by SDF-1 suppressed TLR4 activation by LPS. (B) Effect of Pertussis toxin on activation of TLR4 by LPS. To determine which signals from CXCR4 inhibit activation of TLR4 by LPS, HEK/TLR4(+) cells were transfected with NF κ B- and control-luciferase plasmids and treated with 0.1 μ g/ml pertussis toxin (PT), which inhibits CXCR4 signaling by modifying receptor-associated G β . Controls included HEK/TLR4(+) cells treated with the pertussis toxin B oligomer (PT-B), which binds to cells but does not inhibit CXCR4 signaling. Pertussis toxin did not enhance but instead inhibited LPS-stimulated HEK/TLR4(+) activation. These results indicate that signaling by CXCR4 inhibits activation of TLR4 by LPS, and this effect is likely mediated by the $\beta\gamma$ component of CXCR4.

TLR4 than does LPS, as previous work suggests [4]. Alternatively, the added soluble heparan sulfate might elute SDF-1 from its cellular attachments, primarily heparan sulfate proteoglycans on the cell surface [29], and prevent the SDF-1-stimulated TLR4-inhibitor function.

If signaling by CXCR4 suppresses activation of TLR4 by LPS, we reasoned that interference with CXCR4 signaling should enhance HEK/TLR4(+) activation by LPS. To test this possibility, we treated HEK/TLR4(+) cells with pertussis toxin, a protein that inhibits signaling of G proteins such as G_i associated with CXCR4 [30], and tested responses of the cells to LPS. As Fig. 3B shows, pertussis toxin did not enhance HEK/TLR4 activation by LPS, rather treatment of HEK/TLR4(+) cells with pertussis toxin diminished TLR4 activation by LPS. Since inhibition of CXCR4-associated G_i by pertussis toxin did not increase activation of TLR4 by LPS, the inhibitory signal delivered by CXCR4 is likely mediated by the $\beta\gamma$ subunit of this heterotrimeric G-protein coupled receptor.

The findings presented here indicate that interaction of SDF-1 and CXCR4 with TLR4 raises the threshold for activation of inflammatory cells by TLR4. This change in threshold does not prevent activation of TLR4 by “large” amounts of LPS, but it may prevent inadvertent activation by trace amounts, as may occur at tissue sites remote from the site of infection. Our findings may explain why local infections with Gram negative bacteria usually do not cause the systemic manifestations of sepsis. Since the CXCR4 expression did not change the threshold for activation of TLR4 by heparan sulfate, other local controls must exist for this agonist. Consistent with that possibility, we have recently found that activation of TLR4 by heparan sulfate is suppressed by intact extracellular matrix and relieved by degradation of matrix by proteases (unpublished observations).

CXCR4 may coordinate a delicate balance between promoting and inhibiting inflammation. SDF-1 acting on CXCR4 directs migration of CD3⁺ lymphocytes into areas of inflammation, and recent evidence demonstrates that this pro-inflammatory property of SDF-1 is silenced by proteases secreted by activated neutrophils [31]. The results we report suggest that SDF-1 and CXCR4 may also limit inappropriate inflammatory cues by raising the activation threshold for TLR4. The interplay between the pro- and anti-inflammatory properties of SDF-1, CXCR4, and TLR4 offers new targets to potentially manipulate immune function.

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